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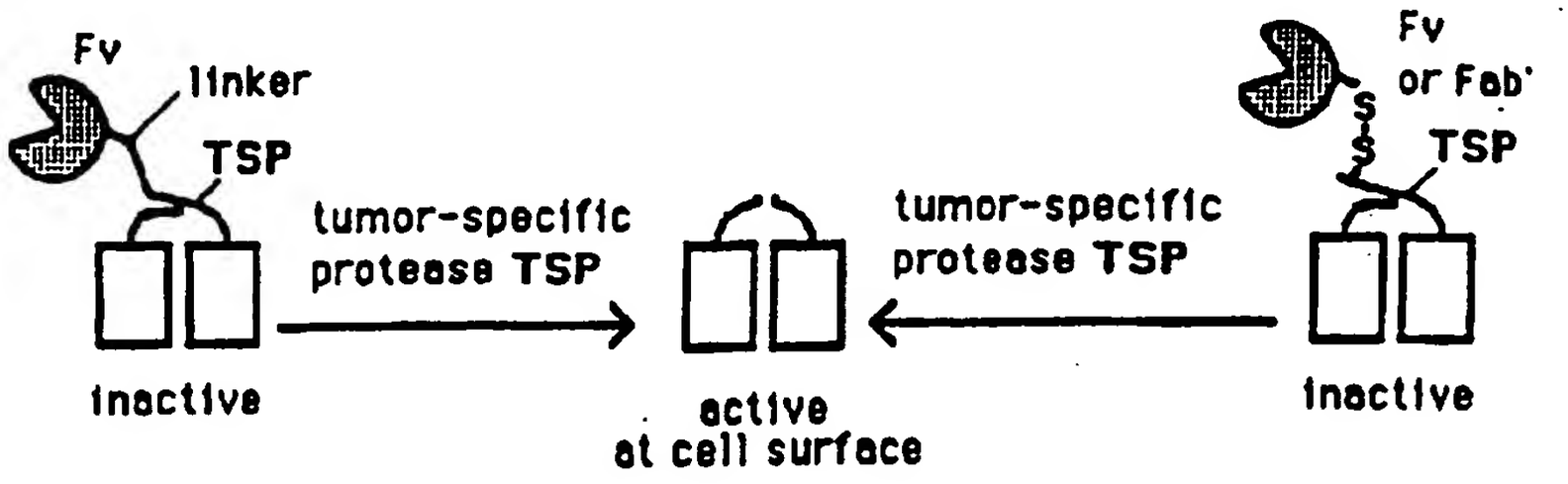


(57) Abstract

An inactive pore-forming agent which is activated to lytic function by a condition such as pH, light, heat, reducing potential, or metal ion concentration, or substance such as a protease, at the surface of a cell.

TRIGGERED PORE-FORMING AGENTS

Field of the Invention

This invention relates to pore-forming compounds.

Background of the Invention

Transmembrane channels or pores can be formed by certain bacterial exotoxins (Bhakdi et al., Philos. Trans. R. Soc. Lond. B. Biol. Sci. 306:311-324, 1983). Pore-forming toxins, such as staphylococcal α-toxin (αHL), assemble into supramolecular amphiphilic polymers in the lipid bilayer of the cell membrane, thus generating stable transmembrane pores.

αHL, a single polypeptide chain of 33,200 daltons, is a water-soluble toxin secreted by *Staphylococcus aureus* (Gray et al., Infect. Immun. 46:615, 1984). αHL is capable of lysing erythrocytes *in vitro* by forming heptameric structures in the membranes of these cells (Gouaux et al., 1994, Proc. Natl. Acad. Sci. USA 91:12828-12831).

Summary of the Invention

Despite developments in surgery, radiation therapy and chemotherapy, safe and effective treatments for many cancers has been elusive. Particularly recalcitrant to treatment are metastatic cells that remain after surgery or radiation therapy. These cells are often resistant to conventional chemotherapy.

The invention addresses this problem by providing a pore-forming compound which is active at the surface of the target cell. Limited pore formation can result in permeabilization of the cell membrane which can improve uptake of substances which are normally difficult to deliver into the cytoplasm of cells, such as cytotoxic chemotherapeutic agents or nucleic acids. Extensive pore formation can itself result in the destruction of the

The two components of the chimeric compound are linked via a non-covalent or covalent bond, or both. Preferably the linkage is a covalent bond involving a sulfur atom, more preferably a disulfide, thioester or thioether bond. The compound of the invention can also be produced as a recombinant fusion protein with the two components of the chimera linked by a peptide bond.

The invention also features a mutant pore-forming agent that is inactive, but able to be converted to an active lytic form by conditions or substances at the surface of a target cell as well as a chimeric compound comprising such an activatable agent linked to a cell-specific ligand. Such a molecule may be activated physically, chemically or biochemically. Physical conditions capable of activating the compound of the invention include but are not limited to the presence of heat or light. The inactive pore-forming agent may also be activated by a conformational change induced by the act of the inactive pore-forming agent binding to its ligand on the surface of the target cell. Chemical conditions capable of activating the pore-forming agent include a change in pH, reduction potential or concentration of metal ions. Biochemical substances, specifically associated with the target cell and capable of activating the compound, include but are not limited to proteases, esterases, glycosidases, ectokinases, or phosphatases. These substances or conditions at the cell surface may be endogenous, e.g., secreted by the target cell, such as a tumor protease, or exogenous, e.g. provided by a source other than the target cell, such as a light emitted from a lamp or fiber-optic device. For example, a therapeutic treatment can comprise topical application of a compound or agent of the invention to a skin tumor and activation by exposure to a light source.

pore-forming agents have been made by modifying αHL with BPNA. An active single-cysteine mutant of αHL is inactivated by reaction with BNPA. When the CNB group, which is introduced by BNPA treatment, is removed by photolysis, pore-forming activity is restored.

Also within the invention is an inactive pore-forming agent that is activated by an enzyme. An enzyme-activated pore-forming agent preferably contains an amino acid extension in an internal domain, e.g., in a glycine-rich loop of an internal domain, of the agent. By the term "internal domain" is meant a domain of a pore-forming agent which does not contain either the amino-terminus or carboxy-terminus of the protein. By the term "glycine-rich loop" is meant a portion of a pore-forming protein 10 to 100 amino acids in length, at least 10% of which are the amino acid glycine. For example, the glycine-rich loop of αHL spans amino acids 111 to 150 and is 20% glycine. By amino acid extension is meant the addition of a peptide of one or more amino acids to the pore-forming agent The amino acid extension contains at least one amino acid, preferably at least 10 amino acids, more preferably at least 40 amino acids, and most preferably, the extension contains a cell-specific ligand, e.g., the antigen-binding domain of an antibody. Preferably, the amino acid extension is cleaved off by the enzyme to restore lytic activity to the pore-forming agent.

The enzyme-activated pore-forming agent of the invention may be an overlap mutant of staphylococcal α-toxin. In preferred embodiments, the enzyme is endoproteinase Lys-C (endo C), the amino acid extension contains a lysine residue, and the agent is K8A(1-142•132-293) or K8A(1-172•132-293). Alternatively, the enzyme is clostripain, the amino acid extension contains an arginine residue, and the agent is K8A,K131R(1-

L-sarcolysin, chlorambucil, hexamethylmelamine, thiotepa, busulfan, carmustine, lomustine, semustine, streptozocin, dacarbazine, methotrexate, fluorouracil, cytarabine, mercaptopurine, thioguanine, pentostatin, vinblastine, vincristine, etoposide, teniposide, actinomycin D, daunomycin, doxorubicin, bleomycin, plicamycin, mitomycin, cisplatin, mitoxantrone, hydroxyurea, procarbozine, mitotane, aminoglutethimide, prednisone, hydroxyprogesterone, diethylstilbestrol, tamoxifen, flutamide, or leuprolide, as well as a DNA oligonucleotide, which is complementary to an essential gene of a cell and capable of eliminating or down-regulating expression of such a gene, or a ribozyme which can disrupt protein synthesis of a cell. A peptide-nucleic acid (PNA), i.e., a peptide backbone substituted with nucleic acid bases (Wittung et al., 1994, Nature 368:561-563), may also be co-administered. PNA binds to DNA more tightly than DNA oligonucleotides, in some cases causing strand displacement on double-stranded DNA.

The invention also features a method of screening pore-forming compounds for the ability to be activated by a condition or substance associated with a target cell. The candidate compound can be generated by combinatorial mutagenesis of a site involved in interaction with a cell-specific condition or substance. Preferably, target cells are contacted with a candidate compound in the presence and absence of a cell-specific condition or substance and cytolysis evaluated as an indication that the candidate compound is activatable.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

component polypeptides separated using sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The left panel shows untreated individual chains and the right panel shows individual chains treated with endo C (1 μg added to 6 μL IVTT mix).

Fig. 4 is a photograph of an SDS-PAGE gel showing the results of treatment of the two-chain mutants with endo C. The left panel shows untreated two-chain mutants and the right panel shows two-chain mutants treated with endo C (1 μg added to 12 μL IVTT mix). The two-chain mutants are converted to fragments that co-migrate with polypeptides 1-131 and 132-293. These fragments are resistant to further breakdown. In the cases of mutants with forward overlaps, a full-length αHL polypeptide is also generated.

Fig. 5 is a schematic interpretation of the proteolysis data. The final state of both forward and reverse overlap mutants is boxed.

Fig. 6 is a photograph of a SDS-PAGE gel showing the time course of proteolysis of the overlap mutant K8A (1-172)•(132-293) with endo C (right). Proteolysis results in the formation of two chains, 1-131 (open arrow) and 132-293 (closed arrow) without further degradation. IVTT mix (12.5 μL), produced using (^{35}S)methionine at 1200 Ci/mmol, was treated with endo C (1.0 μg) at 30°C. Aliquots were removed after designated times and treated with 1 mM Nα-p-tosyl-L-lysine chloromethyl ketone (TLCK). Proteolysis of single-chain K8A was performed as a control (left).

Fig. 7 is a graph showing selective proteolytic activation of αHL 1-172•132-293 overlap mutants containing Lys-131 or Arg-131. Rabbit erythrocyte hemolysis was monitored by the decrease in light scattering at 600 nm after the addition of IVTT mix to a suspension of rabbit erythrocytes. The left panel shows

330 μM (based on amino acid analysis) in 10 mM NaP_i, pH 8.5.

Fig. 15 is a photograph of an autoradiogram showing the chemical modification of αHL-S3C. Modifications by the charged reagents result in characteristic gel shifts after extended electrophoresis. ^{35}S-labeled S3C was treated and subjected to electrophoresis in a 12% SDS polyacrylamide gel. An autoradiogram of a region between the 29.5 kDa and 45.5 kDa molecular weight markers is shown. Lane 1, untreated S3C; lane 2, S3C treated with 4-acetamido-4′-((iodoacetyl)amino)stilbene-2,2′-disulfonate (IASD); lane 3, S3C treated with BNPA; lane 4, BNPA adduct of S3C photolysed at pH 6.0; lane 5, BNPA adduct of S3C photolysed at pH 6.0 after the photolysis products were further treated with IASD; lane 6, BNPA adduct of S3C photolysed at pH 8.5; lane 7, BNPA adduct of S3C photolysed at pH 8.5 after the photolysis products were further treated with IASD. "S3C" represents αHL-S3C; "S3C-CNB" represents S3C adduct formed by reaction of Cys-3 with BNPA; "S3C-ASD" represents S3C adduct formed by reaction of Cys-3 with IASD.

Fig. 16 is a photograph of an autoradiogram showing chemical modification of αHL-R104C. ^{35}S-labeled R104C was treated and subjected to electrophoresis in a 12% SDS polyacrylamide gel. An autoradiogram of a region between of the 29.5 kDa and 45.5 kDa markers is shown. Lane 1, R104C; lane 2, R104C treated with IASD; lane 3, R104C treated with BNPA; lane 4 R104C treated with BNPA and further treated with IASD; lane 5, BNPA adduct of R104C after removal of excess reagent; lane 6, BNPA adduct of R104C after removal of excess reagent photolysed at pH 6.0; lane 7, BNPA adduct of R104C after removal of excess reagent; lane 8, BNPA adduct of R104C

Excess reagent was removed by gel filtration. αHL-R104C-CNB was irradiated at 300 nm (•) or left untreated (o). Hemolytic activity (lysis of rabbit erythrocytes) was measured by monitoring light scattering at 595 nm. The concentration of R104C in the assay was 2.0 μg/ml.

Compounds of the invention

As described in the Summary above, the compound of the invention employs several components which will now be discussed in greater detail.

One component of the compound of the invention is a lytic pore-forming agent, which can be naturally-occurring or synthetically-made. The pore-forming agent can be a molecule as well as a fragment, derivative or analog of such a molecule, which is capable of generating one or more transmembrane pores in the lipid bilayer of a cell which results in the lysis or permeabilization of the cell. Such pore-forming agents derived from bacteria include αHL, *E. coli* hemolysin, *E. coli* colicin, *B. thuringenis* toxin, aerolysin, perfringolysin, pneumolysin, streptolysin O, and listeriolysin. Eucaryotic pore-forming agents capable of lysing cells include defensin, magainin, mellitin, complement, perforin, yeast killer toxin and histolysin. Synthetic organic molecules, such as Pederson's crown ethers and valinomycin, which are capable of forming a lytic pore in a cell membrane can also be used. Other synthetic lytic pore-forming agents are described in Regen et al., Biochem. Biophys. Res. Commun. 159: 566-571, 1989.

The compound of the invention can also include fragments of naturally-occurring or synthetic pore-forming agents which exhibit lytic activity. In addition to substantially full-length polypeptides, the present invention provides for biologically active fragments of the polypeptides. A pore-forming polypeptide or fragment

Modifications (which do not normally alter primary sequence) include *in vivo*, or *in vitro* chemical derivatization of polypeptides, e.g., acetylation, or carboxylation. Also included are modifications of glycosylation, e.g., those made by modifying the glycosylation patterns of a polypeptide during its synthesis and processing or in further processing steps, e.g., by exposing the polypeptide to enzymes which affect glycosylation e.g., mammalian glycosylating or deglycosylating enzymes. Also included are peptides which have phosphorylated amino acid residues, e.g., phosphotyrosine, phosphoserine, or phosphothreonine or have been modified to add fatty acids.

The invention also includes analogs in which one or more peptide bonds have been replaced with an alternative type of covalent bond (a "peptide mimetic") which is not susceptible to cleavage by peptidases. Where proteolytic degradation of the peptides following injection into the subject is a problem, replacement of a particularly sensitive peptide bond with a noncleavable peptide mimetic can result in a more stable peptide and thus, in most cases, a more useful therapeutic agent. Such mimetics, and methods of incorporating them into polypeptides, are well known in the art. Similarly, the replacement of an L-amino acid residues is a standard way of rendering the polypeptide less sensitive to proteolysis. Also useful are amino-terminal blocking groups such as t-butyloxycarbonyl, acetyl, theyl, succinyl, methoxysuccinyl, suberyl, adipyl, azelayl, dansyl, benzyloxycarbonyl, fluorenylmethoxycarbonyl, methoxyazelayl, methoxyadipyl, methoxysuberyl, and 2,4,-dinitrophenyl. Although most modifications are designed to make proteins more resistant to proteolytic degradation, the invention also embraces modifications which enhance such degradation, for the purpose of

or other techniques of molecular biology, such as polymerase chain reaction (PCR). Also included are analogs which include residues other than naturally occurring L-amino acids, e.g., D-amino acids, non-naturally occurring or synthetic amino acids, e.g., β or γ amino acids, or L-amino acids with non-natural side chains using known methods. Methods for site-specific incorporation of non-natural amino acids into the protein backbone of proteins are described in Ellman et al., Science 255:197, 1992. The peptides of the invention are not limited to products of any of the specific exemplary process listed herein.

Useful mutants can be identified using the inventive screening assay, in which a combinatorial library containing a semi-random mutational cassette is screened for activity or the ability to be activated by a condition or substance.

Cell-specific ligands

The delivery portion or cell-specific ligand of the compound can be any ligand which binds specifically to the target cell. The invention can employ not only intact monoclonal or polyclonal antibodies, but also an immunologically-active antibody fragment, such as a Fab' or $(Fab')_2$ fragment, or a genetically engineered Fv fragment (Ladner et al., U.S. Patent No. 4,946,788).

Delivery agents can also include other cell-specific ligands, e.g., hormones such as steroid hormones, or peptide hormones; neuroactive substances, e.g., opioid peptides; insulin; growth factors, e.g., epidermal growth factor, insulin-like growth factor, fibroblast growth factor, platelet derived growth factor, tumor necrosis factor; cytokines, e.g., an interleukin (IL), e.g., IL-2, IL-4, or IL-5; melanocyte stimulating hormone; a substance or receptor which has affinity for a

Proteins can be chemically modified by standard techniques to add a sulfhydryl group. For example, Traut's reagent (2-iminothiolane-HCl) (Pierce Chemicals, Rockford, IL) can be used to introduce a sulfhydryl group on primary amines, such as lysine residues or N-terminal amines. A protein or peptide modified with Traut's reagent can then react with a protein or peptide which has been modified with reagents such as N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) or succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) (Pierce Chemicals, Rockford, IL).

A free sulfhydryl group of an antibody may be generated using methods known to the art. For example, the antibody can be enzymatically cleaved with pepsin to yield $(Fab')_2$ fragments, which are then gently reduced with dithiothreitol (DTT) or 2-mercaptoethanol to yield free sulfhydryl-group-containing Fab' fragments. Antibody fragments, e.g., single chain Fv, can also be expressed recombinantly and genetically engineered to contain a terminal cysteine group using methods known to the art or chemically modified as described above.

Once the correct sulfhydryl groups are present on each component of the compound, the two components are purified, sulfur groups on each component are reduced; the components are mixed; and disulfide bond formation is allowed to proceed to completion at room temperature. To improve the efficiency of the coupling reaction, the cysteine residue of one of the components, e.g., cysteine-αHL, can be activated prior to addition to the reaction mixture with 5,5'-dithiobis(2-nitrobenzoic) acid (DTNB) or 2,2'-dithiopyridine, using methods known to the art. Following the reaction, the mixture is dialyzed against phosphate buffered saline to remove unconjugated molecules. Sephadex chromatography or the like is then

for the purging of unwanted leukemic cells from blood by transiently exposing blood to light via a UV-emitting extracorporeal device. Accessible tumors such as skin cancers can also be treated in this manner, and relatively inaccessible tumors, such as lung cancers, can be reached using light emissions from fiber optic devices.

Chemical conditions, such as pH, reducing potential or the presence of metal ions, may also serve as activators. For example, the compound of the invention can be modified to contain a protecting group which is altered or removed by exposure to a chemical condition at the surface of the target cell, thus resulting in the activation of lytic activity.

Since metastatic cancer cells have been shown to secrete matrix metalloproteinases (Liotta et al., Cell 64:327-336, 1991), a metalloproteinase recognition site that has been incorporated into the compound of the invention can be acted upon by the enzyme at the surface of a tumor cell, resulting in the activation of pore-forming function. The compounds of the invention which are activated by metalloproteinases can be used to prevent vascularization of tumors. The process of active vascularization at a tumor site is associated with the secretion of specific proteases by tumor cells. These proteases can activate the pore-forming function of re-engineered inactive pore-forming agents of the invention, thereby preventing the vascularization process (which is required for growth and metastasis) or damaging tumor cells at the tumor site.

In another example, metal ions or chelating agents, e.g., ethylenediaminetetraacetic acid (EDTA), may be infused into the animal systemically or directly into the site of the target cell to activate or deactivate lytic function.

Since some proteins and polypeptides may be more difficult to inactivate than others, a more highly charged or more bulky sulfhydryl-directed protecting group based on the 2-nitrobenzyl functionality may be required. BPNA may be altered (while retaining the 2-nitrobenzyl functionality) consistent with these requirements. For example, the size of the protecting group may be increase by substituting the aromatic ring with an alkyl group. Alternatively, a large polymer, e.g., polyethylene glycol (PEG), can be linked to the aromatic ring to increase the size of the protecting group.

The reactivity of BPNA may also be altered by replacing the bromide leaving group with iodide or yet more reactive leaving groups.

The chiral center in BNPA that must yield diastereomeric cysteine adducts could also be a disadvantage in some circumstances and might be remedied without too much difficulty, e.g., by separating the R and S isomers of BPNA using a "chiral" HPLC column.

Modification of sulfhydryl groups with BPNA

Known reagents for introducing photoremovable functional groups, e.g., 2-nitrobenzyl chloride, were found to be too insoluble in aqueous buffers to permit efficient modification of proteins and polypeptides under most circumstances. Therefore, BNPA was made and used to modify proteins and polypeptides by introducing a α-carboxy-2-nitrobenzyl (CNB) protecting group. BNPA can be produced in high yield by the bromination of 2-nitrophenylacetyl chloride, followed by hydrolysis of the acyl chloride group (see Fig. 12). BNPA is highly water-soluble at pH values around neutral. The α-carboxy group also increases the reactivity of the electrophilic center towards the cysteinate anion. Further, the

Therapeutic administration of re-engineered pore-forming agents

The compound of the invention can be administered to an animal, e.g., a human, suffering from a medical disorder, e.g., cancer, or other conditions characterized by the presence of a class of unwanted cells. For example, therapeutic destruction of CD8-bearing T cells in HIV-infected patients may be efficacious in restoring a proper ratio of CD4 to CD8 cells in such patients (Rennie, Sci. Amer. 5/93:24-24).

The amount of compound administered will vary with the type of disease, extensiveness of the disease, and size of species of the animal suffering from the disease. Generally, amounts will be in the range of those used for other cytotoxic agents used in the treatment of cancer, although in certain instances lower amounts will be needed because of the increased specificity of the compound. The compound of the invention may be used in combination therapy in which the compound of the invention is administered either simultaneously or sequentially with a therapeutic agent which is not easily internalized by the target cell, such as cytotoxic chemotherapeutic agents, described above. For example, an oligomeric anti-sense DNA can be used to eliminate or down-regulate the expression of genes necessary for cell survival. Permeabilization of target cells by the compound of the invention can also facilitate the entry of ribozymes into the target cell, which can kill the cell by disrupting protein synthesis.

Generally, the compound of the invention will be administered by intravenous infusion, although it may also be administered subcutaneously or injected directly into site at which unwanted cells are to be destroyed, e.g. a tumor site. For example, inactive pore-forming agents which are activated by the enzymes endo C and

αHL mutants

Mutants of αHL were generated from the plasmid pT7-NPH8S, which encodes the wild-type sequence of αHL as secreted by *Staphylococcus aureus*. pT7-NPH8S was made from pT7-NPH8 (Walker et al, J. Biol. Chem. 268:21782, 1992) by using oligonucleotide-directed mutagenesis to correct the mutation, Ser-217→Asn, that occurred during an earlier PCR. The codon Lys-8 in pT7-NPH8S was then changed to Ala by oligonucleotide-directed mutagenesis to eliminate an unwanted protease recognition site.

Truncated αHL genes were generated from the K8A gene by PCR using procedures described in Walker et al., *supra*. To obtain overlap mutant K8A,K131R(1-172•132-293), K8A,K131R was made from K8A by oligonucleotide-directed mutagenesis and then K8A,K131R1-172) was generated from K8A,K131R by PCR.

Coupled IVTT

Full length recombinant αHL (Walker et al., J. Biol. Chem. 267:10902-10909, 1992), truncated recombinant αHL polypeptides (Walker et al., J. Biol. Chem. 267:21782-21786, 1992), and recombinant two-chain complementary αHL polypeptides (Walker et al., J. Biol. Chem. 268:5285-5292) were then produced by IVTT according to methods known in the art by using an *E. coli* S30 extract (Promega No. L4500). The mix was supplemented with T7 RNA polymerase (NEB No. 251L, added at 2000 U/mL) and rifampicin (20 μg/mL) and (^{35}S)methionine. For hemolysis assays, the final methionine concentration in the IVTT mix was 0.5 mM (0.8 Ci/mmol) to ensure the synthesis of mutant polypeptides at concentrations in the range 10-50 μg/mL. Synthesis was carried out for 60 min. at 37°C. The integrity of the translation products and their relative concentrations were evaluated by SDS-PAGE and autoradiography.

Laemmli, *Nature* 227:680, 1970). Radiolabelled markers (Gibco BRL) were (^{14}C)methylated proteins; myosin heavy chain (M_r=200,000); phosphorylase b (M_r=97,400); bovine serum albumin (M_r=68,000); ovalbumin (M_r=43,000); carbonic anhydrase (M_r=29,000); β-lactoglobulin (M_r=18,400); lysozyme (M_r=14,300).

Pore Formation

IVTT was carried out in the presence of (^{35}S)methionine (1200 Ci/mmol) and the reaction was stopped by the addition chloramphenicol (100 μM) and unlabelled methionine (5 mM), which prevent the incorporation of ^{35}S into rabbit erythrocyte membrane proteins. IVTT mix (5 μL), untreated or treated with endo C (1 μg), was incubated with 10% rabbit erythrocytes (50μL) for 60 min. at 20°C in K-PBSA. The cells or membranes were recovered by centrifugation, dissolved in 30 μL 1X loading buffer (U.K. Laemmli, *Nature* 227:680, 1970), warmed at 45°C for 5 min. and subjected to electrophoresis in a 12% SDS-polyacrylamide gel.

Characterization of αHL mutants

Staphylococcal α-hemolysin, a lytic pore-forming toxin, has been remodeled yielding inactive molecules that can be activated by Lys/Arg-directed proteases, which inactivate the wild-type protein. Wild type αHL polypeptides with nicks near the midpoint of the central glycine-rich loop (Walker et al., *supra*) are held together by a domain-domain interaction and are hemolytically active. By contrast, mutant αHL proteins comprising two αHL truncation mutants that overlap in the central loop (overlap mutants) have no or greatly reduced pore-forming activity. Overlap mutants have now been designed that are activated when redundant amino acids in the loop are removed by proteases.

Selective activation by endo C and clostripain

The requirement for protease specificity was tested by altering the recognition sequence in the central loop. Two overlap mutants, 1-142•132-293 and 1-172•132-293, were produced from the double point mutant K8A,K131R, and compared with the same overlap mutants generated from K8A. The Lys-131-containing mutants were selectively activated by the lysine-directed endo C and the Arg-131 mutants selectively activated by the arginine-directed clostripain (Fig. 7).

Selective activation by cathepsin B

Two-chain constructs of αHL, 1-131•119-293 and 1-172•132-293 have been made for activation by the tumor protease, cathepsin B. Both constructs contain the following mutations: Lys 8 → Ala, Gly130 → Arg, and Lys 131 → Arg. These re-engineered inactive αHL molecules were treated with cathepsin B *in vitro*. Activation was evaluated by: (1) analyzing the proteolytic cleavage products using SDS-PAGE; and (2) measuring the induction of lytic activity, i.e., lysis of rabbit erythrocytes. Data from these experiments indicate that these re-engineered αHL molecules are inactive until activated by cathepsin B.

Activation of these molecules can also be induced by treatment with tumor cell extracts, e.g., extracts of B16F10, a melanoma tumor cell line which secretes cathepsin B.

The inactive two-chain constructs of αHL can be further mutagenized and screened to identify mutants that are optimally activated by the activating protease, as described below.

the target cell. Pore-forming agents which can be photoactivated using the methods described below can be used for the permeabilization of selected cells in a collection of cells and for the permeabilization of a predetermined region of the surface of a single cell.

Modification of reduced glutathione (GSH) with BNPA

Stock solutions of the reduced form of glutathione (GSH, 100 mM in water) and BNPA (100 mM in 100 mM NaP_i, pH 8.5) were freshly made and used to prepare a reaction mixture containing 20 mM GSH, 20 mM BNPA in 250 mM NaP_i, pH 8.5, which was incubated overnight at room temperature in the dark. TLC (silica gel, system 1: ethanol, water, ammonia 7:2:1) revealed ~80% conversion of GSH to a new compound GS-CNB (Rf 0.70, ninhydrin positive, 254 nm absorbing). GSH (Rf 0.59, ninhydrin positive) and GSSG (oxidized GSH) almost comigrated in this system, but further analysis by system 3 (see below) revealed that the remainder of the GSH (~20%) had been oxidized to GSSG. GS-CNB was purified by preparative TLC (system 1) and isolated in 48% yield based on quantitative amino acid analysis (Hoogerheide et al., Anal. Biochem., 1992, 201:146-51). UV (10 mM NaP_i, pH 8.5): λmax = 265 nm, $\epsilon 4$ 600 (see Fig. 14).

Photolysis of GSH-CNB

GSH-CNB in water (2 mM, 42.5 µL) was buffered by the addition of 500 mM NaP_i (5.0 µL) at either pH 6.0 or pH 8.5, followed by the addition of freshly prepared DTT solution (100 mM in water, 2.5 µL). The mixture was placed on ice in a microtiter plate well and photolyzed at 300 nm, 3.5 cm from a Fotodyne UV illuminator (Foto UV 300). Samples (6 µL) were removed at 0, 15, 30, 60, 90, and 120 min. and spotted onto a TLC plate, which was eluted with ethanol/water/ammonia (8:1:1, system 2) or

150 μL with 100 mM Tris.HCl containing 10 mM DTT so that the concentration of DTT was 1 mM. The final concentration of BNPA and its low mass sulfhydryl adducts was ≤ 20 μM. The modified proteins were stored at -20°C before use.

BNPA modification of staphylococcal αHL-R104C

αHL-R104C (0.36 mg/mL) was dialyzed against 10 mM Tris.HCl, pH 8.5, containing 5 mM β-ME. Dialyzed R104C (100 μL), 10 mM DTT in water (20 μL) and 1.0 M Tris.HCl, pH 8.5 (40 μL) were incubated for 5 min. at room temperature before the addition of 100 mM BNPA in 100 mM NaP_i, pH 8.5 (40 μL). After 3 h at room temperature, 1.0 M DTT (5 μL) was added and excess reagents were removed from the protein by gel filtration on Bio-Gel P-2 (Bio-Rad) eluted with 10 mM Tris.HCl, pH 8.5, containing 50 mM NaCl. A fraction was desalted and the buffer exchanged for 100 mM Tris.HCl, pH 8.5, 1.0 mM DTT by ultrafiltration (Amicon, Microcon-3)

Photolysis of CNB-αHL

CNB-αHL in 100 mM Tris.HCl (pH 6.0 or 8.5) containing 1 mM DTT (60, μL) was placed in a well of a 96-well microtiter plate and irradiated for 30 min. on ice through a 285 nm cut-off filter (Oriel, #51220) at 3.5 cm from a Foto UV 300 illuminator (Fotodyne). To assay for the unmasking of the protected cysteine residue, samples of the irradiated αHL polypeptides were treated with IASD and analyzed by SDS-PAGE. For the pH 8.5 sample, irradiated αHL (5 μL) was diluted with 100 mM Tris.HCl, pH 8.5 (3 μL), and reacted with 100 mM IASD in water (2 μL) for 1 h at room temperature. For the pH 6.0 sample, irradiated αHL (5 μL) was diluted with 1.0 M Tris.HCl, pH 8.5 (2 μL) and water (1 μL) and treated in the same way.

The photochemistry of 2-nitrobenzyl derivatives is highly dependent upon pH. In this case, a higher yield of GSH (~50%) was achieved when irradiation was carried out for the optimal time (e.g., 30 min.) at pH 6.0, rather than at pH 8.5 (~30%). Oxidation products of GSH, GSSG (oxidized GSH) and GSO_3H (glutathionesulfonic acid), were not detected after 30 min. of irradiation.

Modification and photodeprotection of a model polypeptide: αHL-S3C

BNPA was used to selectively modify αHL with a single cysteine residue (αHL-S3C). After modification of the polypeptide, deprotection, i.e., removal of the CNB group, of αHL-S3C by near-UV light was evaluated. Prior to BNPA modification, gel-shift electrophoresis was used to determine sulfhydryl accessibility in single-cysteine mutants of αHL. Radiolabeled αHL polypeptides were modified with IASD and subjected to SDS-PAGE.

αHL-S3C was chosen as a model for studying polypeptide modification with BNPA because αHL-S3C undergoes a particularly large reduction in electrophoretic mobility upon modification with IASD (Krishnasastry et al., FEBS Lett., 1994, 356:66-71), thus facilitating analysis. Reaction of radiolabeled αHL-S3C with BNPA was found to produce a substantial gel shift upon prolonged electrophoresis (Fig. 15, lane 3). Because the BPNA-shift is less pronounced, it is distinguishable from the shift produced by IASD (Fig. 15, lane 2).

Complete modification of αHL-S3C was achieved with 20 mM BNPA at pH 8.5 after 1 h at room temperature. Excess reagent was removed by repeated dilution and ultrafiltration. The modified hemolysin, αHL-S3C-CNB was then irradiated at pH 6.0 or pH 8.5 under conditions that had been previously established to give optimal release

polypeptide that both flanks the central loop and interacts with residues near the N terminus that cooperate with the loop in the final step of pore formation.

αHL-R104C is as active as WT-αHL before modification, and upon modification with IASD, gives an especially pronounced gel shift and has negligible activity. After treatment with BNPA, the pore-forming activity was again lost. The modification of αHL-R104C with BNPA and subsequent activation (deprotection) with near-UV irradiation was examined by SDS-PAGE, as described for αHL-S3C.

Important differences were seen between the behavior of αHL-R104C and αHL-H3C. Reaction with BNPA produced the expected decrease in electrophoretic mobility (Fig. 16, lane 3), but, at best, only 80% conversion to αHL-R104C-CNB was obtained. It appeared that unmodified αHLR104 remained after the reaction; however, close examination of the gels and treatment of the BNPA reaction products with IASD (Fig. 16, lane 4) revealed that there were two side-reaction products that migrated close to αHL-R104C, but contained no modifiable sulfhydryl groups. The same result was obtained with highly purified unlabeled R104C obtained by expression in *S. aureus*. Since these reaction products appeared to be devoid of hemolytic activity, the photochemistry of BNPA-modified αHL-R104C was examined.

BNPA-modified αHL-R104C was irradiated at 300 nm at pH 6.0 or pH 8.5. In contrast to αHL-S3C, more αHLR104C was generated at pH 8.5 than at pH 6.0 (Fig. 16, lanes 6 and 8), and chain cleavage to a product with M_r ~30000 was less prevalent. The polypeptide presumed to be αHL-R104C was shifted to reduced mobility after treatment with IASD, demonstrating the presence of a free sulfhydryl group (Fig. 16, lanes 9 and 10). The large

Although the action of photoactivated αHL-R104C-CNB may be too slow (see Fig. 7) for certain applications such as the production of very rapid local transmembrane fluxes of Ca^{2+}, it is adequate for other applications. For example, αHL-R104C-CNB can be used to permeabilize cells to cause an exchange of small molecules, i.e., therapeutic agents which are about 2-3 kDa in size, across a cell membrane. Other lytic agents which create larger pores, e.g., aerolysin, perfringolysin, pneumolysin, streptolysin O, and listeriolysin, can be modified according to the invention to make photoactivatable pore-forming agents which can permeabilize cells with pores large enough to allow protein molecules to pass.

Since the removal of the 2-nitrobenzyl group from modified proteins or polypeptides is rapid, BPNA-modification can be used to make other photoactivatable pore-forming agents with different, i.e., faster, lysis kinetics. For example, lytic agents such as aerolysin, perfringolysin, pneumolysin, streptolysin O, listeriolysin, *Bacillus thuringensis* toxin, *E. coli*-derived hemolysin, *E. coli*-derived colicin, defensin, magainin, mellitin, complement, perforin, yeast killer toxin, or histolysin, can be modified according to the invention to render them inactive until photoactivated to lytic activity. These data suggest that upon irradiation with near-UV light, the inactive photoactivatable pore-forming agents of the invention are activated and permeabilize cell membranes. Water-soluble synthetic pore-forming compounds, e.g., lytic peptides or synthetic organic molecules, can also be modified using BPNA.

The modified α-hemolysin and other pore-forming agents modified according to the invention can be used for the permeabilization of one cell in a collection of cells (e.g. a neuron in a network) or one aspect of a single cell (e.g. the presynaptic terminus of a neuron). Other applications include nanostructure synthesis, e.g., two-dimensional arrays of pores with patterned open and closed states.

While rabbit erythrocytes undergo colloid osmotic lysis when treated with αHL, most other cells do not (Bhakdi et al., 1993, Med. Microbio. Immunol. 182:167-175). Hence, αHL-R104C-CNB and related BPNA-modified hemolysins can be used for cell permeabilization. Permeabilization with pore-forming agents compares favorably with other techniques for introducing molecules into cells, including microinjection and electroporation. The pore-forming agents of the invention have the advantages of being clinically useful, i.e., they can be delivered to remote areas of the body and activated, e.g., with a fiber optic device, and controllable, i.e., they can be triggered or switched on and off *in vivo*.

Since αHL binds irreversibly to membranes and is unlikely to diffuse from one cell to another, selective permeabilization of one cell in a collection of cells can be accomplished using photoactivatable αHL molecules, e.g., αHL-R104C-CNB. However once bound to a cell surface, αHL may diffuse on the surface of a cell. Surface-bound αHL diffusion will define the time interval for which permeabilization can be confined to one aspect of a cell, thereby allowing the exchange of reagents into defined regions of the interior. Since the effective activity of the pore would be greatly reduced by diffusional dilution, manipulation of this parameter represents one way of decreasing or shutting down activity.

phosphorylation can be permanently activated with a thiophosphate group. The resulting thiophosphopeptides or proteins can then be modified with BPNA to render them photoactivatable.

Non-pore-forming proteins or polypeptides can be mutated using cysteine scanning mutagenesis as described above for αHL. Prior to BPNA-modification of cysteine mutants of αHL, pore-forming activity was examined before and after modification with the bulky, dianionic reagent IASD. Data from this study permitted the selection of potential candidate cysteine mutants that would likely be inactivated by the less drastic modification brought about by BNPA treatment. BNPA modification can be carried out directly on the large sets of cysteine mutants that are available for several polypeptides. The extent of IASD modification can be readily evaluated by charge-shift electrophoresis. Alternatively, since substantial gel shifts occur with BPNA-modified proteins and polypeptides, the effects of BPNA modification can be evaluated directly. If necessary, the extent of modification with BNPA can be determined by subsequent treatment with IASD.

The applications for caged peptides are numerous, e.g., the local release of enzyme inhibitors at specific sites within cells and the unmasking of peptides that block specific protein-protein interactions. BNPA can also be used to protect cysteines in peptides produced by chemical synthesis after they have been fully deprotected, as demonstrated by the modification of glutathione (described above) as well as to block cysteines or cysteine analogs that are placed in large synthetic peptides by chemical ligation. Unnatural amino acids with reactive sidechains introduced by chemical synthesis or site-specific modification can also be modified or protected with BNPA.

Construction of Ab-αHL conjugates

Any protease, which is specifically associated with a particular cell type, can be employed as an activator of inactive compounds of the invention, e.g., plasminogen activator, specifically urokinase-type plasminogen activator (uPA), which has been specifically associated with the metastatic phenotype of cancer cells (Liotta et al., Cell 64:327-336, 1991). The recognition sequence of plasminogen activator, Gly-Arg, or the same sequence preceded by a negatively charged amino acid, the recognition sequence for uPA, can be incorporated into the compound of the invention for the purpose of specific activation of the pore-forming agent. Similarly, the autocatalytic cleavage site for human collagenase IV (CIV) (Stetler-Stevenson et al., J. Biol. Chem. 264:1353-1356, 1989), can also be incorporated into the compound. These recognition sites can also serve as the basis for the semi-random combinatorial mutagenic cassette used in the generation of novel lytic pore-forming agents, described below.

Mutant two-chain αHL can be constructed as shown in Fig. 9. In the construct shown, the protease site for trypsin has been added. Since murine B16 melanoma cells have been shown to secrete plasminogen activator and collagenase IV (Reich et al. *Res.* 48:3307-3312, 1988; Wang et al., *Cancer Res.* 40:288-292, 1980), the protease cleavage sites for plasminogen activator and collagenase IV (gelatinase/MMP2) (Stack et al. *Arch. Biochem. Biophys.* 287:240-249, 1991; Stack et al. *J. Biol. Chem.* 264:4277-4281, 1989; Harrison et al. *Anal. Biochem.* 180:110-113, 1989) can also be added for specific activation by this cell type. Linker regions can be glycine- and serine-rich peptides.

A reduced Fab fragment or an Fv fragment with an engineered cysteine can also be linked to an activated

collagenase IV and secretions from cells producing such enzymes.

Activation on the surface of target cells

(i) Target cells; DNP-human erythrocytes: Active wild type αHL efficiently lyses rabbit erythrocytes, which bear αHL receptors, but not human erythrocytes and other cells which do not bear receptors for αHL. The surfaces of human erythrocytes, which are resistant to αHL-mediated lysis, must first be modified with dinitrophenyl fluoride (DNP-F) to make them susceptible to lysis. The derivatized cells can then be treated with anti-DNP-K-αHL, washed, and then incubated with trypsin. In this example, the anti-DNP antibody serves to direct the compound to the derivatized target cells, resulting in increased local concentration of αHL polypeptides on the surface of cells which triggers the assembly of pores.

(ii) DNP-B16 melanoma cells: B16 melanoma cells can also be modified with DNP-F and then treated with anti-DNP-PA-αHL or anti-DNP-CIV-αHL. In this case, target cell viability can be assayed *in vitro* over 3 days by standard assays including trypan blue exclusion, ^{51}Cr release, [^{35}S]methionine uptake (protein synthesis) and [^{3}H]thymidine uptake (DNA replication) as an indication of the ability of the compound to permeabilize the target cells.

B16-specific antibody linked to αHL, e.g., anti-B16-PA-αHL or anti-B16-CIV-αHL, can be used to direct the compounds of the invention to the surface of B16 melanoma cells which have not been treated with DNP. Cell viability after incubation with the compound of the invention can be assayed as described above.

Example 5: Screens for activatable pore-forming lytic agents

A novel screening technique to identify lytic pore-forming agents which can be activated by cell-associated substances or conditions will now be described.

Protease site selection by combinatorial mutagenesis:

A novel screening technique, based on combinatorial mutagenesis, has been devised for determining the peptide sequence specificity of proteases secreted by tumors and, at the same time, obtaining lytic pore-forming agents that are more rapidly and selectively activated by these proteases. These mutants can be then be used as cytotoxic agents against the same cells (Fig. 11). For example, mutant αHLs can be screened from combinatorial libraries on the basis of their ability to be activated by target cell extracts to lyse rabbit erythrocytes. A mutagenic cassette, containing random nucleic acid sequences generated by methods well known in the art, can be incorporated into the plasmid encoding the pore-forming agent. Since the aspects of the specificity of certain relevant proteases is known, the engineered protease sites encoded by the mutagenic cassette can be based on these sequences and need not be completely random.

For example, candidate clones from a combinatorial plasmid library can be plated on replicate nitrocellulose filters. The bacterial colonies on the filters are then lysed and recombinant proteins allowed to bind to the filters. One replicate filter is contacted with a cell extract of a target cell and as a control, a duplicate filter remains untreated. Both filters are exposed to blood agar plates and scored for the appearance of hemolytic plaques. Alternatively, the filters may be

Following the identification of a putatively positive colony, the plasmid can then be purified from the bacterial cells according to methods well-known in the art and analyzed to determine the DNA sequence of the mutagenic cassette. The candidate pore-forming compound can be expressed by IVTT and subjected to further analysis. The ability of the candidate pore-forming compound to be activated by the target cell can further be confirmed by incubating the translation product in the presence and 4absence of target cell extract, adding target cells, and assaying target cell lysis.

Selection of metal-sensitive mutants

A similar strategy using the screening assay of the invention can also be used to identify mutants with metal binding capabilities. As above, the screen can be based on a semi-random mutagenic cassette since complexity will be limited by using Cys or His codon-based oligonucleotides. As above, protein from bacterial colonies expressing αHL can be transferred to nitrocellulose filters in the presence and absence of metal ions. The duplicate filters are then placed in contact with blood agar plates and the blood agar plates scored for the appearance of hemolysis. The ability of metal ions to block or activate hemolysis can thus be evaluated.

Other embodiments are within the following claims.

What is claimed is:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Pro Leu Arg Pro
1

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Ser Ile Arg Cys
1

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Leu Pro Arg Lys
1

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids

CLAIMS:

1. An inactive pore-forming agent which is activated by a condition or substance at the surface of a cell.

2. The agent of claim 1, wherein said agent is selected from the group consisting of staphylococcal α-toxin, aerolysin, perfringolysin, pneumolysin, streptolysin O, listeriolysin, *Bacillus thuringensis* toxin, *E. coli*-derived hemolysin, *E. coli*-derived colicin, defensin, magainin, mellitin, complement, perforin, yeast killer toxin, and histolysin.

3. The agent of claim 1, wherein said condition is a change in pH, light, heat, reducing potential, or metal ion concentration.

4. The agent of claim 3, wherein said condition is light.

5. The agent of claim 4, wherein said agent comprises at least one cysteine linked to an α-carboxy-2-nitrobenzyl (CNB) group which is removed from said agent by irradiation at a wavelength of at least 300 nm, thereby activating said agent.

6. The agent of claim 5, wherein said agent is a single cysteine mutant of αHL.

7. The agent of claim 6, wherein said mutant is selected from the group consisting of R104C, E111C, K168C, and D183C.

8. The agent of claim 7, wherein said agent is R104C.

19. The agent of claim 16, wherein said enzyme is clostripain and said extension comprises an arginine residue.

20. The agent of claim 19, wherein said agent is K8A,K131R(1-142•132-293) or K8A,K131R(1-142•132-293).

21. The agent of claim 16, wherein said enzyme is a cell-specific protease.

22. The agent of claim 21, wherein said protease is tumor-specific.

23. The agent of claim 22, wherein said protease is cathepsin B.

24. The agent of claim 23, wherein said agent is K8A,G130R,K131R(1-172•132-293).

25. The agent of claim 23, wherein said agent is K8A,G130R,K131R(1-131•119-293).

26. A metal-sensitive pore-forming agent, wherein lytic activity of said agent is regulated by a change in metal ion concentration.

27. The agent of claim 26, wherein said agent comprises an amino acid substitution in an internal domain of said agent.

28. The agent of claim 27, wherein said agent is inactivated by a divalent metal ion and activated by a chelating agent.

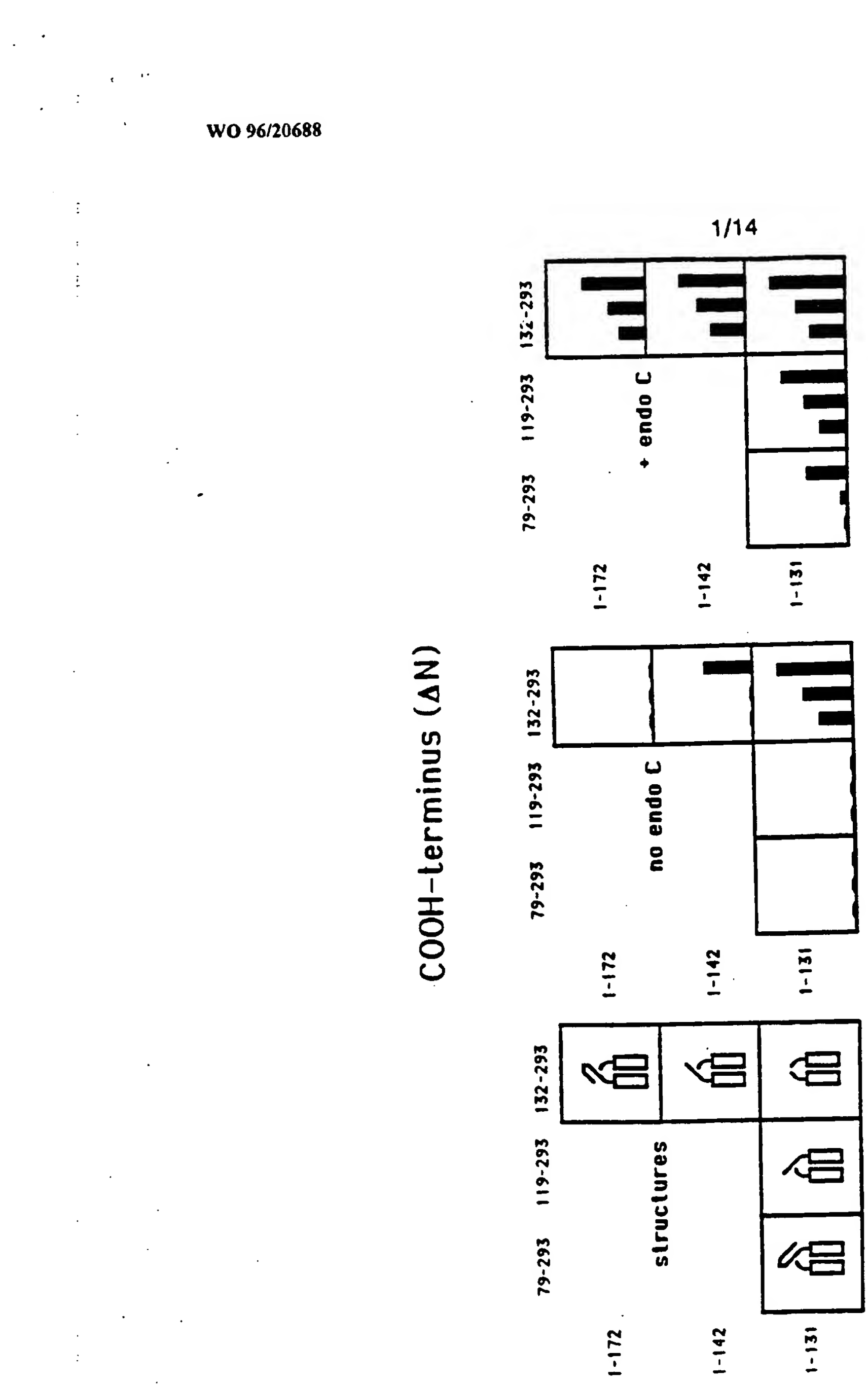

COOH-terminus (ΔN)
132-293
119-293
79-293
+ endo C
1-172
1-142
1-131
132-293
119-293
79-293
no endo C
1-172
1-142
1-131
132-293
119-293
79-293
structures
1-172
1-142
1-131
NH2-TERMINUS (ΔC)

FIG. 1

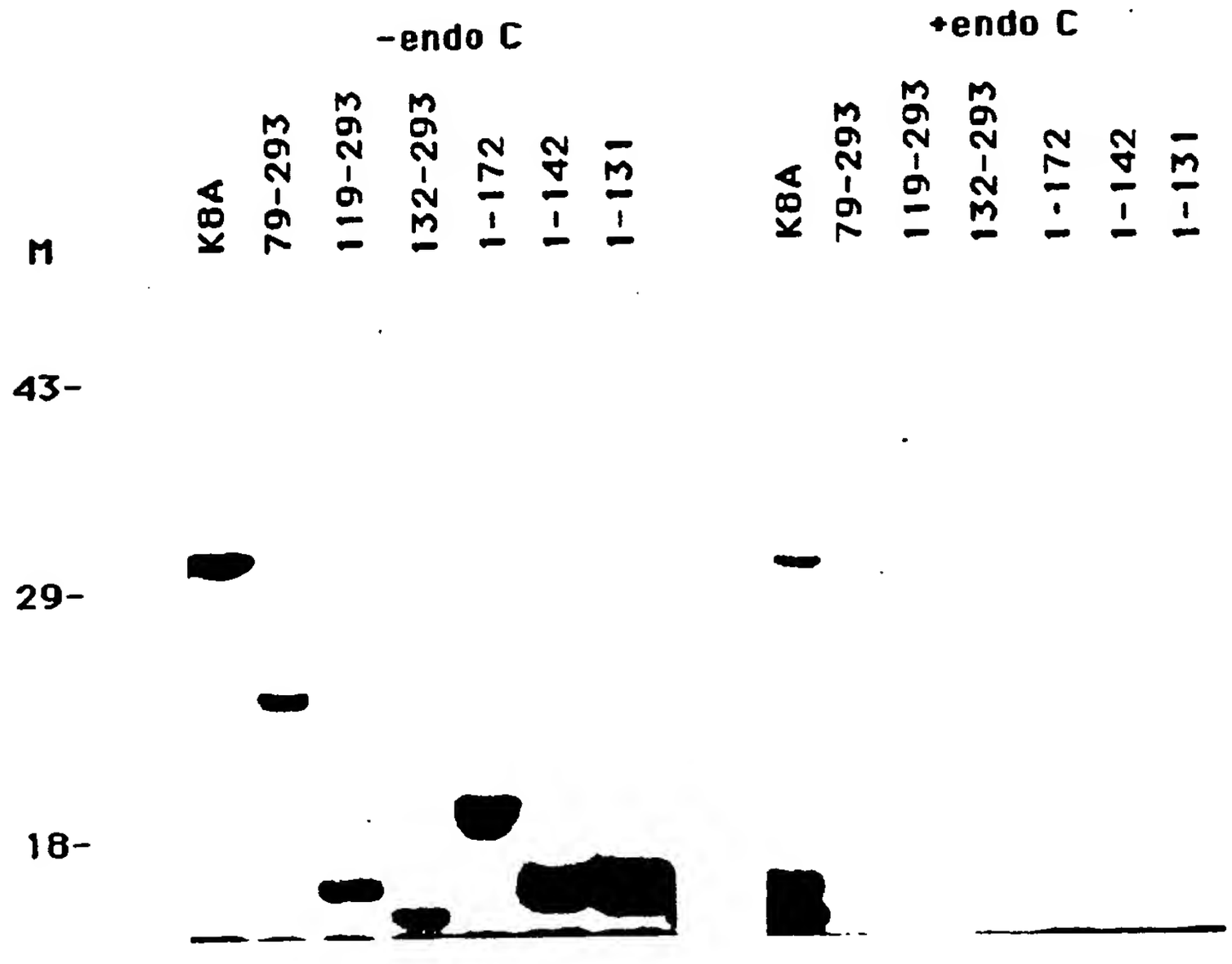


FIG. 3

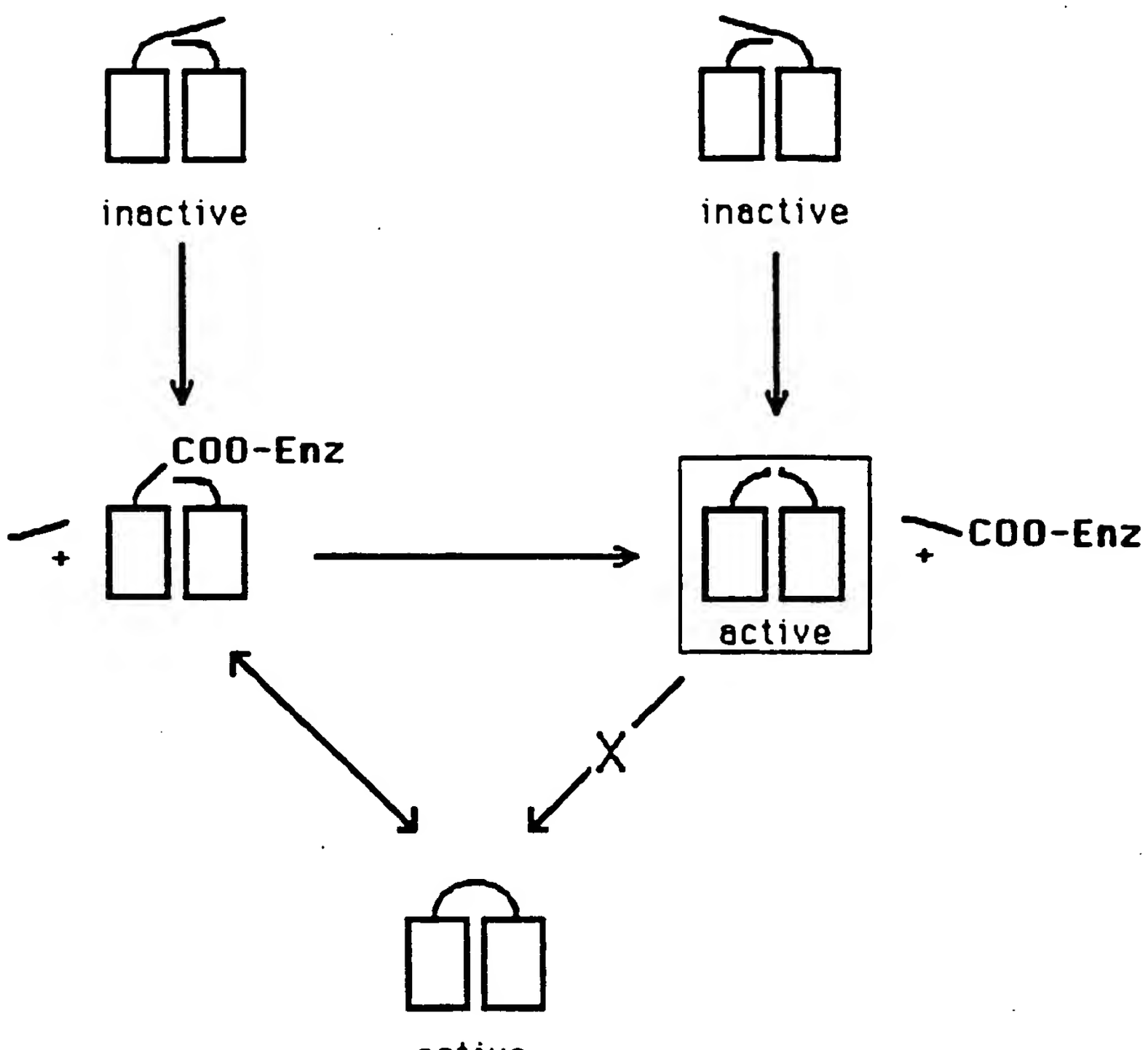
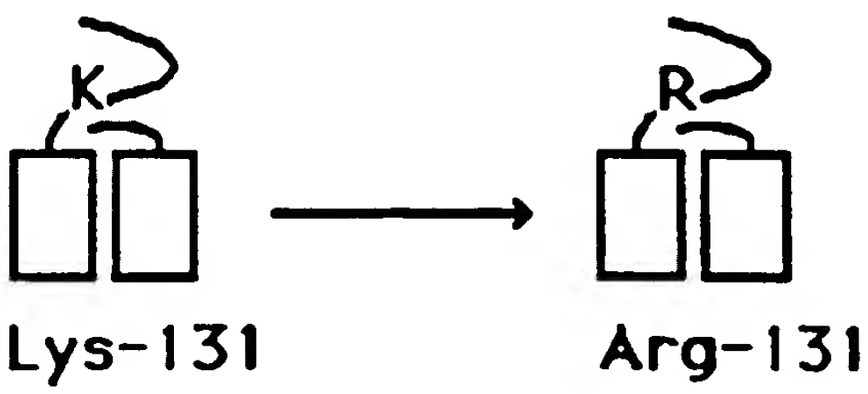
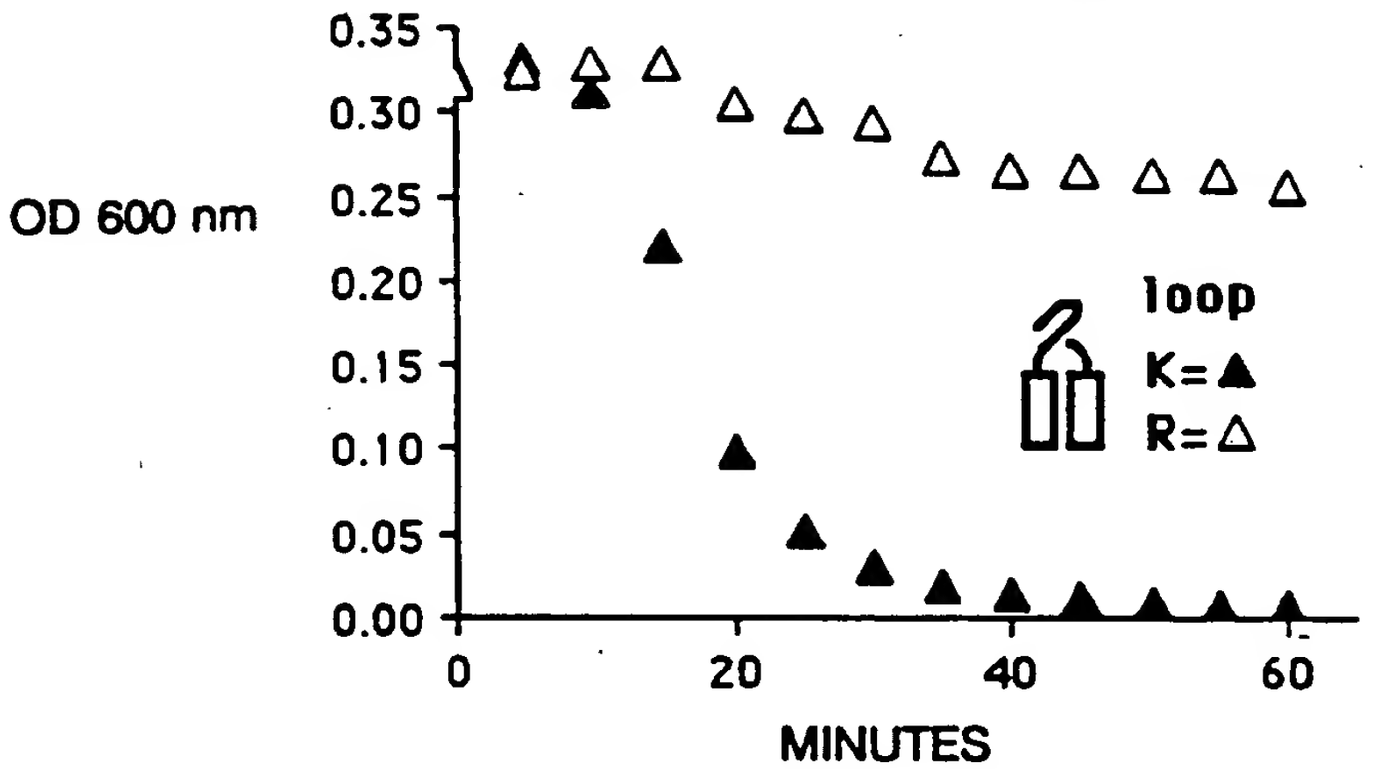


FIG. 5


K
R
Lys-131
Arg-131


+ endo C
OD 600 nm
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
20
40
60
MINUTES
loop
K=▲
R=△

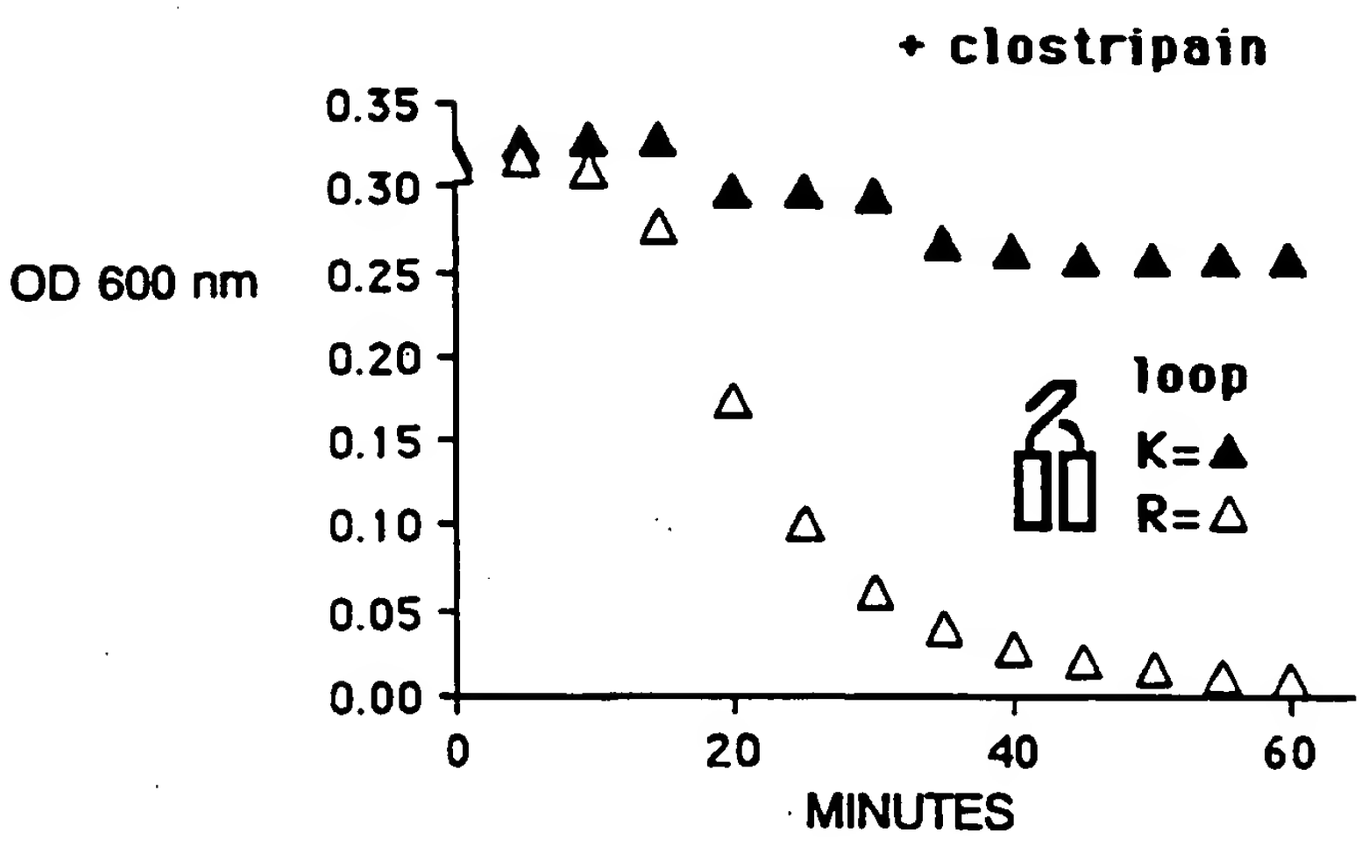

+ clostripain
OD 600 nm
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
20
40
60
MINUTES
loop
K=▲
R=△

FIG. 7

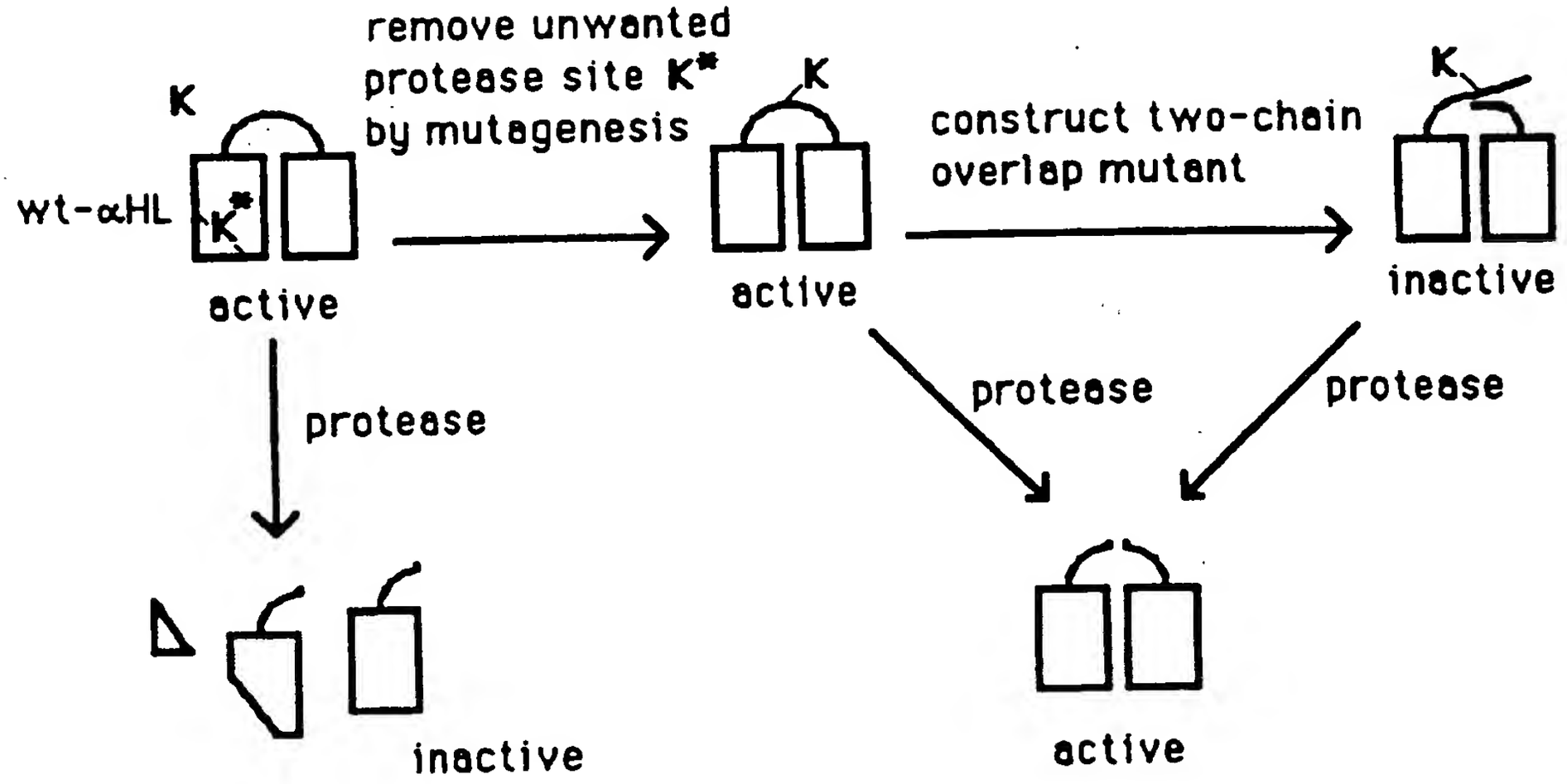

remove unwanted
protease site K*
by mutagenesis
construct two-chain
overlap mutant
K
K
K
wt-αHL
K*
active
active
inactive
protease
protease
protease
inactive
active

FIG. 9

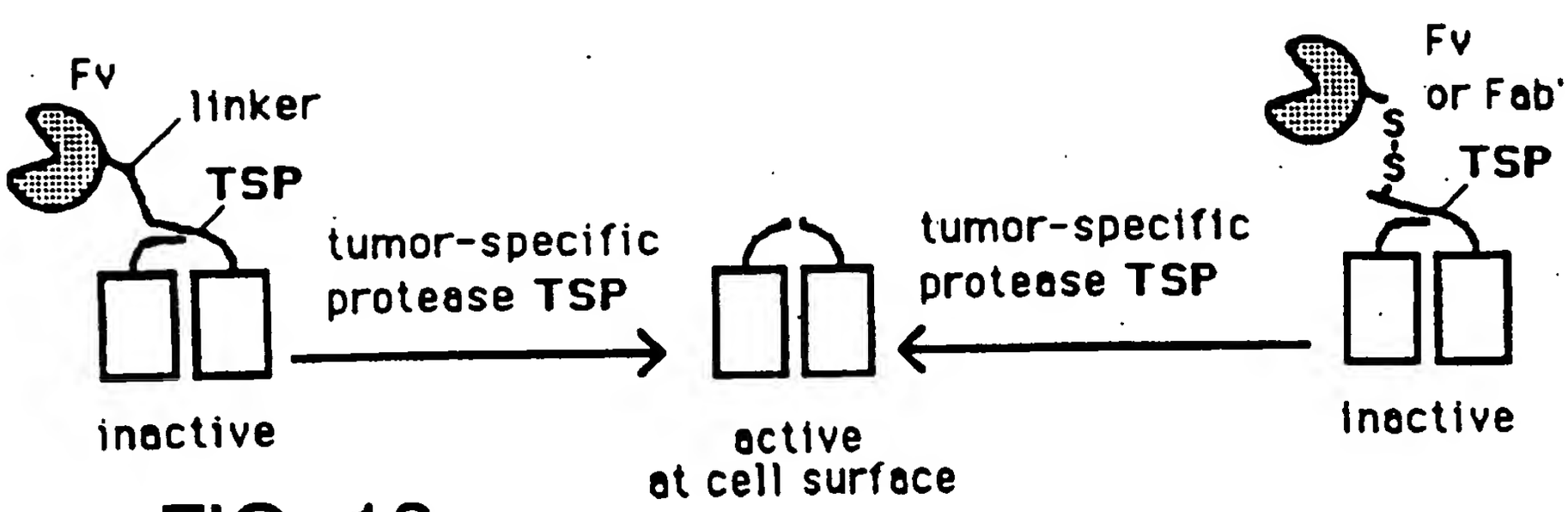

Fv
linker
TSP
tumor-specific
protease TSP
tumor-specific
protease TSP
Fv
or Fab'
S-S
TSP
inactive
active
at cell surface
inactive

FIG. 10

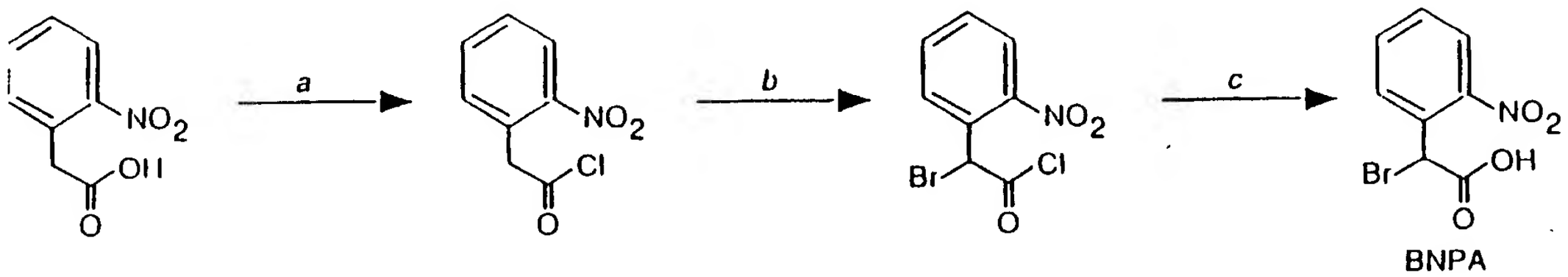


FIG. 12

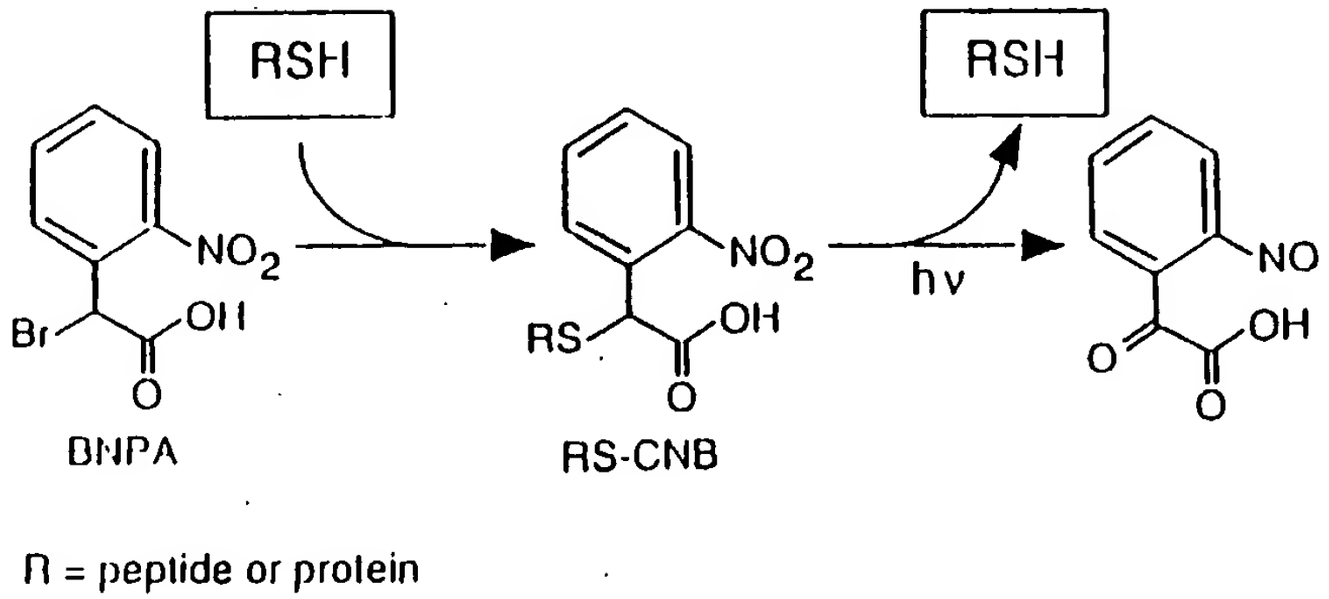


FIG. 13

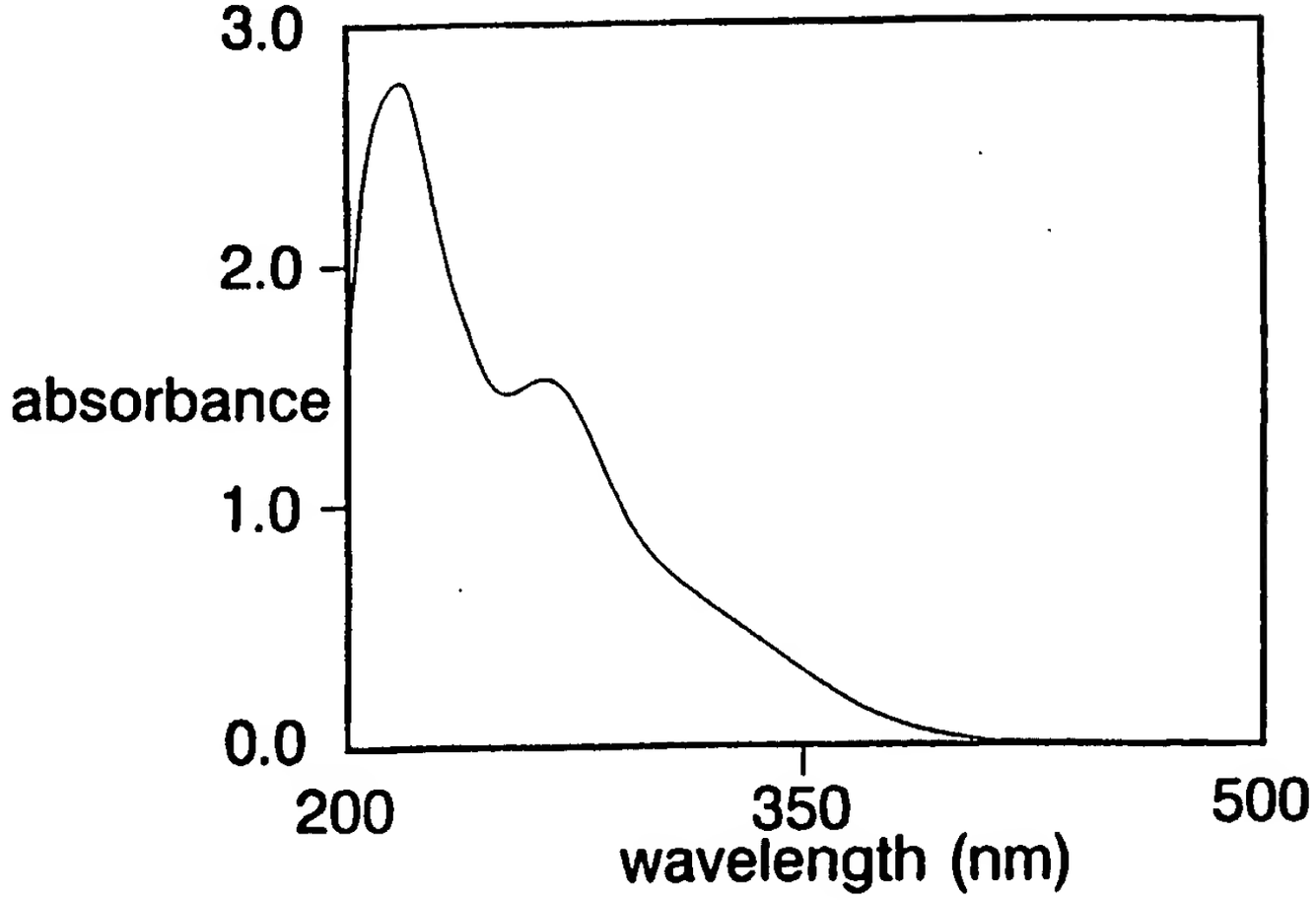


FIG. 14

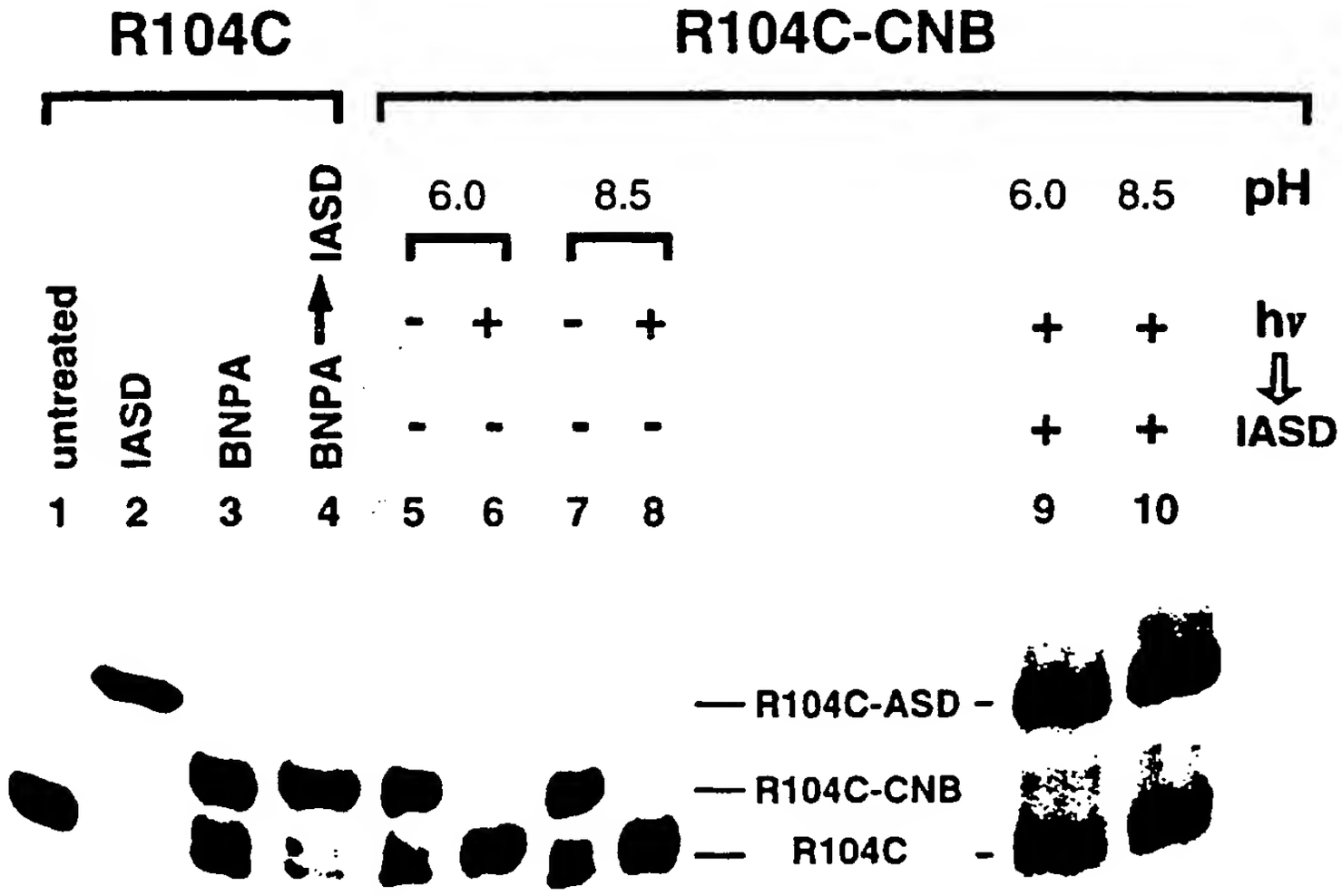

R104C
R104C-CNB
6.0
8.5
6.0
8.5
pH
IASD
untreated
IASD
BNPA
BNPA
hν
IASD
1 2 3 4 5 6 7 8 9 10
R104C-ASD
R104C-CNB
R104C

FIG. 16

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International Bureau

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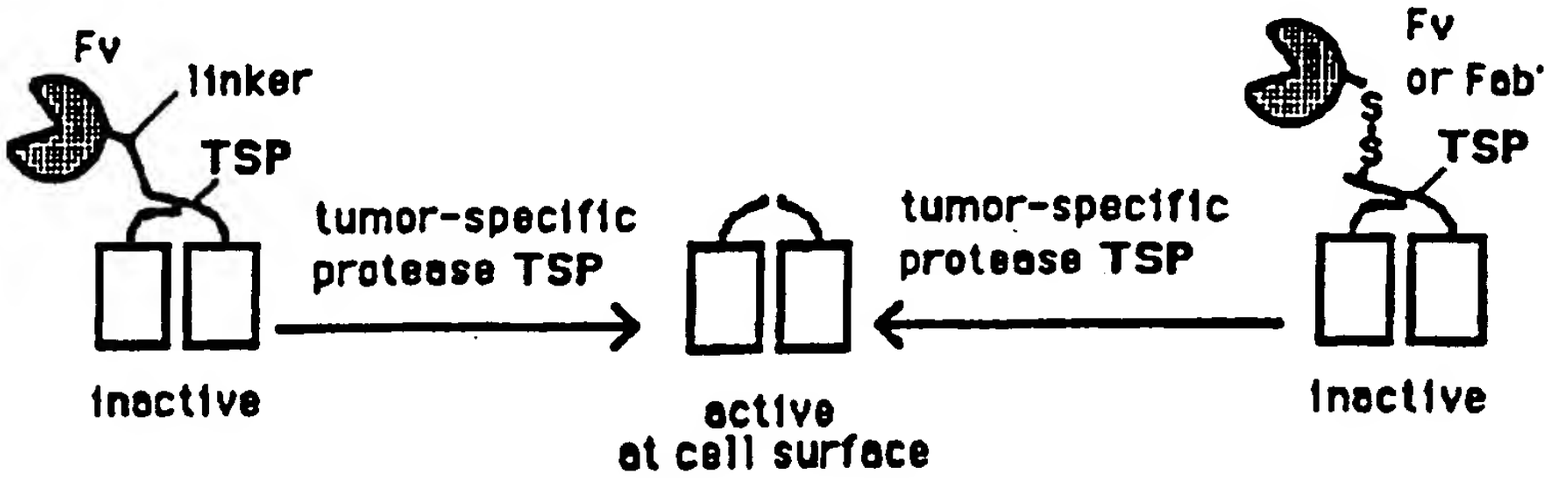

(57) Abstract

An inactive pore-forming agent which is activated to lytic function by a condition such as pH, light, heat, reducing potential, or metal ion concentration, or substance such as a protease, at the surface of a cell.

INTERNATIONAL SEARCH REPORT

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PCT/IB95/01164

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07K 19/00; G01N 15/08
US CL : 530/387.3; 435/7.2

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/387.3; 435/7.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS/MEDLINE, BIOSIS, LIFESCI, EMBASE, WPI

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PROTEIN ENGINEERING, VOL.7, NO. 1, ISSUED 1994, B. WALKER ET AL., "A PORE-FORMING PROTEIN WITH A PROTEASE ACTIVATED TRIGGER", PAGES 91-97, SEE ENTIRE DOCUMENT.	1-32
Y	SCIENCE ET AL., VOL. 255, ISSUED 07 FEBRUARY 1992, I. AMATO, "MOLECULAR DESIGN GETS INTO A HOLE", PAGE 684, SEE ENTIRE DOCUMENT.	1-32
Y	WO, A, 94/25616 (WORCHESTER FOUNDATION FOR EXPERIMENTAL BIOLOGY) 10 NOVEMBER 1994, PAGES 1-31, SEE ENTIRE DOCUMENT.	1-32

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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Date of the actual completion of the international search	Date of mailing of the international search report
06 JUNE 1996	12 JUL 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer CHRISTOPHER EISENSCHENK